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PCR testing can be as accurate as culture for diagnosis of *Ichthyophonus hoferi* in Yukon River Chinook salmon *Oncorhynchus tshawytscha*

Toshihide Hamazaki[1,*], Eryn Kahler[1], Bonnie M. Borba[2], Tamara Burton[1]

[1]Alaska Department of Fish and Game, Division of Commercial Fisheries, 333 Raspberry Road, Anchorage, Alaska 99518, USA
[2]Alaska Department of Fish and Game, Division of Commercial Fisheries, 1300 College Road, Fairbanks, Alaska 99701, USA

ABSTRACT: We evaluated the comparability of culture and PCR tests for detecting *Ichthyophonus* in Yukon River Chinook salmon *Oncorhynchus tshawytscha* from field samples collected at 3 locations (Emmonak, Chena, and Salcha, Alaska, USA) in 2004, 2005, and 2006. Assuming diagnosis by culture as the 'true' infection status, we calculated the sensitivity (correctly identifying fish positive for *Ichthyophonus*), specificity (correctly identifying fish negative for *Ichthyophonus*), and accuracy (correctly identifying both positive and negative fish) of PCR. Regardless of sampling locations and years, sensitivity, specificity, and accuracy exceeded 90%. Estimates of infection prevalence by PCR were similar to those by culture, except for Salcha 2005, where prevalence by PCR was significantly higher than that by culture ($p < 0.0001$). These results show that the PCR test is comparable to the culture test for diagnosing *Ichthyophonus* infection.

KEY WORDS: Parasite · Diagnostic · Accuracy · Sensitivity · Specificity · Genetic · Amplification

INTRODUCTION

In epidemiological studies, accurate assessment of infection prevalence is imperative, and thus using the most accurate diagnostic method is preferred. In practice, however, choice of method is also based on other considerations, such as logistics of sampling and specimen handling, cost and speed of diagnosis, and the objectives of the assessment. For Chinook salmon *Oncorhynchus tshawytscha* migrating back to the Yukon River (Alaska, USA), determining infection of prevalence of *Ichthyophonus hoferi* (hereafter referred to as *Ichthyophonus* because of taxonomic uncertainties of strains) is the primary management concern (Kocan et al. 2004). *Ichthyophonus* is a highly pathogenic and lethal parasite that infects many marine and anadromous fishes and is known to cause outbreaks and mass mortality of herring (*Clupea* spp.; McVicar 1999, Kramer-Schadt et al. 2010). A series of studies showed a high infection prevalence (23–40%) at Emmonak, 38 river km (rkm) from the Yukon River mouth, and decline in prevalence (10–15%) at Chena (1472 rkm) and Salcha (1544 rkm) river spawning grounds (Kocan et al. 2004, Kahler et al. 2007, 2011). Current knowledge suggests that Yukon River Chinook salmon are infected by *Ichthyophonus* before entering the river, that *Ichthyophonus* targets primarily heart muscle and spreads over all body parts, and that about 60% of infected fish die before they reach the spawning grounds (pre-spawning mortality; Kocan et al. 2004, 2011).

For assessment of *Ichthyophonus* infection prevalence, several methods have been employed, including macroscopic examination of tissue, histological evaluation, *in vitro* explant culture (McVicar 1999), and polymerase chain reaction (PCR) using *Ichthyophonus*-specific primers (Whipps et al. 2006). Of those, the *in vitro* heart explant culture method is recommended as the diagnostic 'gold standard' (Kocan et al. 2011). However, this culture method requires

*Email: toshihide.hamazaki@alaska.gov

fresh tissue samples to be stored in a refrigerated culture medium and transported to a laboratory within 1 to 3 d, and it takes about 14 d of incubation for diagnosis (Kocan et al. 2011). This method is not practical in the study of Yukon River Chinook salmon, where samples are collected at very remote field sites with no means of refrigerating and transporting culture samples in a timely manner. Further, the prevalence of *Ichthyophonus* needs to be determined within 1 to 3 d of sampling, if it is to be used for fishery management during the salmon run season.

Under these constraints, the PCR method (Whipps et al. 2006) is a viable alternative if its accuracy is comparable to culture. The PCR method has several advantages over culture: (1) samples are stored in 95% ethanol and do not require special incubation and handling, (2) less time is required for diagnosis (~48 h), (3) large numbers of samples can be tested, (4) samples can be tested repeatedly, and (5) morphologically similar strains can be separated. Aside from obvious high costs of operation, the major disadvantage of PCR is not being able to distinguish between live and non-living pathogens, which potentially can produces more false positives (i.e. a host carrying non-living pathogens is diagnosed as 'infected or infectious;' Stanley 2003). This disadvantage, however, is probably not an issue in our study because it is unlikely that Yukon River Chinook salmon carry non-living *Ichthyophonus*.

For equivalency of PCR to culture, Whipps et al. (2006) reported that sensitivity and specificity (correctly identifying fish positive and negative for *Ichthyophonus*) of PCR exceeded 90% but that sensitivity of PCR on lightly infected Chinook salmon dropped to 0 to 50%. While suggesting that PCR is comparable to culture, Whipps et al. (2006, p. 145) stated that 'light infection of *Ichthyophonus hoferi* was not detected as often as heavy infection when using PCR on single tissue.' This is often quoted in a context that PCR underestimates the prevalence of *Ichthyophonus* infections (e.g. Kocan 2009), which we argue is incorrect. Here, we report the accuracy of PCR in diagnosing *Ichthyophonus* infection with larger samples and clarify the results of Whipps et al. (2006) by re-examining their data.

MATERIALS AND METHODS

Field samples

Samples were taken from 2 stages of the spawning migration: the mouth of the Yukon River (Emmonak) and the Chena and Salcha river spawning grounds, for 3 yr during the 2004 to 2006 period (Kahler et al. 2007, 2011; Table 1). Emmonak represents a detection of *Ichthyophonus* at an early (possibly light) infection stage, whereas that at the Chena-Salcha spawning grounds represents the final (possibly severe) stage of infection. At Emmonak, Chinook salmon were captured during the migration period (3 June to 15 July) using 8.5 inch (ca. 21.6 cm) mesh set and drift gillnets, and at the Chena and Salcha river spawning grounds fresh dead carcasses (presumed 2 to 3 d post mortem) were collected every 2 to 3 d throughout the river reach during the spawning period (mid- to late August; Table 1, see Kahler et al. 2007, 2011 for details). From each fish, 2 samples (0.5 g each) of heart muscle tissue were aseptically sampled: 1 sample was aseptically placed into a refrigerated *Ichthyophonus* culture medium and the other was placed into 95% ethanol for PCR. The tissue samples were sent to the Alaska Department of Fish and Game Fish Pathology Laboratory in Anchorage within 2 d and were tested for *Ichthyophonus* immediately.

Diagnosis of *Ichthyophonus* infection

Diagnosis of *Ichthyophonus* was conducted using 2 methods: (1) tissue explant culture (McVicar 1999) and (2) PCR (Whipps et al. 2006). In the culture method, the cardiac muscle tissue was cultured in 7 ml Eagle's Minimal Essential Medium supplemented with 5% fetal bovine serum, 100 IU ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, and 100 µg ml^{-1} gentamicin (*Ichthyophonus* culture medium) and incubated at 14°C for a minimum of 14 d. The cultures were periodically examined microscopically for *Ichthyophonus*. In the PCR method, we followed the protocol of Whipps et al. (2006), with exact proce-

Table 1. *Oncorhynchus tshawytscha*. Sample size, dates, and total number of Chinook salmon sampled from 2004 to 2006. rkm: river km

Location	rkm	Date sampled	Year	n
Emmonak	38	3 Jun–5 Jul	2004	104
		2 Jun–12 Jul	2005	105
		7 Jun–11 Jul	2006	104
Chena	1472	22 Jul–12 Aug	2005	324
		28 Jul–8 Aug	2006	162
Salcha	1544	17 Jul–12 Aug	2005	571
		28 Jul–10 Aug	2006	260

dures described by Meyers (2009). Nucleic acid was extracted from the ethanol-fixed sample following the manufacturers' protocol for the DNeasy Tissue kit (Qiagen). *Ichthyophonus*-specific PCR primers Ich7f (5′-GCT CTT AAT TGA GTG TCT AC-3′) and Ich6r (5′-CAT AAG GTG CTA ATG GTG TC-3′) were used to amplify a 371 base pair fragment of the *Ichthyophonus* small subunit rDNA (Whipps et al. 2006). Reactions were prepared in 25 µl volumes, consisting of 1× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 25 pmol each primer, 0.025 U μl^{-1} *Taq* DNA polymerase, and 2 µl of template DNA. The reactions were carried out for 35 cycles consisting of 94°C for 30 s, 60°C for 45 s, 72°C for 60 s, preceded by an initial denaturation at 95°C for 3 min, and followed by a final extension at 72°C for 7 min. For each PCR run, a minimum of 1 known positive, 1 known negative, and no-template controls were included (Meyers 2009). The products were visualized on an agarose gel stained with ethidium bromide.

Data analysis

Unlike controlled laboratory diagnostic experiments, true infection status of each fish is unknown in field samples, and neither PCR nor culture diagnosis is 100% accurate. Acknowledging this, we calculated sensitivity (ability of a test to identify true positive), specificity (ability to identify true negative), and accuracy (ability to identify true positive and negative) of PCR based on diagnosis from culture as the 'true' infection status.

$$\text{Sensitivity} = \frac{C^+P^+}{C^+P^+ + C^+P^-} \quad (1)$$

$$\text{Specificity} = \frac{C^-P^-}{C^-P^- + C^-P^+} \quad (2)$$

$$\text{Accuracy} = \frac{C^+P^+ + C^-P^-}{\text{Total samples}} \quad (3)$$

where C^+P^+ = number of samples diagnosed positive by both culture and PCR methods; C^+P^- = number of samples diagnosed positive by culture and negative by PCR; C^-P^- = number of samples diagnosed negative by both culture and PCR; C^-P^+ = number of samples diagnosed negative by culture and positive by PCR. If PCR is equivalent to culture for diagnostic capability, we expect that all 3 indices would be close to 100%.

We also compared estimates of infection prevalence by culture and PCR methods using the exact binomial test of homogeneity (H_0) of marginal probabilities (i.e. McNemar test; McNemar 1947).

$$H_0: p_{P^+} = \frac{C^-P^+}{C^-P^+ + C^+P^-} = p_{C^+} = \frac{C^+P^-}{C^-P^+ + C^+P^-} = 0.5 \quad (4)$$

If PCR is less sensitive than culture, we expect that the marginal probability of culture positive (p_{C+}) would be significantly higher than that of PCR (p_{P+}) and that the difference would be larger for Emmonak samples (presumed light infection stage) than for Chena and Salcha samples (presumed heavy infection stage).

RESULTS

In all 3 locations, the majority of fish diagnosed positive by culture were also diagnosed positive by PCR (Table 2). Few fish were diagnosed positive either by PCR or culture only. The exceptions were samples from Chena and Salcha 2005, in which 27 fish tested positive by PCR only (C^-P^+: 10 Chena, 17 Salcha). Sensitivity, specificity, and accuracy of PCR to culture exceeded 90%, except for Emmonak 2006 and Chena 2006, where sensitivity was below 85%. In these 2 cases, the number of culture positive was <20 in which changes of 1 sample greatly influenced (i.e. ±5%) the calculation of sensitivity.

Of the 7 sampling events, 2 cases had higher estimates of infection prevalence for culture than PCR (Emmonak 2006, Chena 2006), whereas 3 cases had higher estimates by PCR than culture (Emmonak 2004, Chena 2005, and Salcha 2005). Of those, only Salcha 2005 was statistically significant ($p < 0.0001$).

DISCUSSION

In comparing the accuracy of one diagnostic test to other tests using field samples, it is important to acknowledge that a diagnostic test is rarely 100% accurate. Hence, even though we defined the results from the culture (i.e. positive or negative) as the 'true' infection status, false positive and false negative results could occur in both PCR and culture tests. Acknowledging this, when both diagnostic tests are equivalent, we expect that (1) both tests show similar estimates of infection prevalence, and (2) sensitivity, specificity, and accuracy are close to 100%.

In all cases, the data showed that PCR is equivalent to culture for diagnosis of *Ichthyophonus* infec-

Table 2. Diagnosis of *Ichthyophonus* infection (95% confidence intervals in parentheses) by culture (C) and PCR (P), sensitivity (SNS), specificity (SPC), and accuracy (ACC) (%) at each sampling site. Superscript indicates positive (+) and negative (–) by corresponding test; n: number of samples in which both culture and PCR tests were conducted successfully

Location	Year	n	Number of samples				Infection prevalence (%)					
			C^+P^+	C^+P^-	C^-P^+	C^-P^-	C	P	p	SNS	SPC	ACC
Emmonak	2004	90	16	0	3	71	17.8 (14.8–20.8)	21.1 (17.7–24.5)	0.25	100	95.9	96.7
	2005	104	23	2	2	77	24 (20.5–27.5)	24 (20.5–27.5)	1	92	97.5	96.2
	2006	104	13	4	0	87	16.3 (13.7–19.0)	12.5 (10.4–14.6)	0.25	76.5	100	96.2
	Total	298	52	6	5	235	19.5 (17.7–21.2)	19.1 (17.4–20.9)	1	89.7	97.9	96.3
Chena	2005	317	32	3	10	272	11 (10.0–12.1)	13.2 (12.0–14.5)	0.09	91.4	96.5	95.9
	2006	155	16	3	1	135	12.3 (10.6–14.0)	11 (9.4–12.5)	0.63	84.2	99.3	97.4
	Total	472	48	6	11	407	11.4 (10.5–12.4)	12.5 (11.4–13.5)	0.33	88.9	97.4	96.4
Salcha	2005	564	56	0	17	491	9.9 (9.2–10.7)	12.9 (12.0–13.9)	0	100	96.7	96.7
	2006	244	25	3	3	213	11.5 (10.2–12.8)	11.5 (10.2–12.8)	1	89.3	99.1	97.5
	Total	808	81	3	20	704	10.4 (9.8–11.0)	12.5 (11.7–13.3)	0	96.4	97.2	97.2

tion and that PCR may be more sensitive than culture as evidenced in Salcha 2005 samples (Table 2). It is unlikely this was caused by false positives of PCR due to field or laboratory contamination (Whipps et al. 2006) because there was no noticeable pattern in occurrence of PCR-positive fish in the sequence of sampling events, except that 11 out of 17 fish (65%) were noted as having excessive fungus on their skin. Those fish might be too old to be tested positive by culture (i.e. dead *Ichthyophonus*). The culture method can reliably isolate *Ichthyophonus* from a postmortem fish for up to 4 d when left at ambient temperature (Kocan et al. 2004); however, the length may change depending on actual field environmental conditions. We are not aware of any study determining postmortem detectability periods for *Ichthyophonus* by PCR. Simultaneously, many fish also arrive at spawning grounds with their skin excessively covered with fungus. In fact, of the 564 Salcha 2005 samples, 268 (48%) were noted as having excessive fungus, and the estimate of *Ichthyophonus* infection prevalence by PCR was significantly higher than by culture in both fungus-covered (PCR: 12.7% versus culture: 8.6%, $p < 0.0001$) and non-fungus-covered (PCR: 13.2% versus culture: 11.1%, $p = 0.003$) fish. The PCR test may be more sensitive than culture in some cases.

Re-analyses of Tanana 2004 data (Whipps et al. 2006)

As noted, our data indicate that the PCR test is equivalent to or could be more accurate than the culture test in diagnosing *Ichthyophonus* infection, which suggests that the 50% sensitivity of PCR on heart tissue from 'lightly infected' fish (Whipps et al. 2006) is more likely an anomaly. Whipps et al. (2006) examined the infection status of a fish with multiple diagnostic methods (histology, PCR, and culture) from multiple tissues (heart, kidney, somatic muscle), and defined 'lightly infected' fish as those that had only 1 tissue diagnosed as positive (excluding results of PCR) and ≤1 spore mm^{-2} tissue found by histology. In Tanana 2004 samples, there were 6 'lightly infected' fish: 3 positive by heart culture (Cases D, E) and 3 positive by muscle culture (Cases F, G) only (Table 3). Of those, 3 fish were positive by heart PCR (Cases D, F), which resulted in a sensitivity of 50% matching Table 3 of Whipps et al. (2006). However, if heart culture were tested in the same way as PCR, its sensitivity is also 50% (i.e. 3 heart culture positive out of 6). In fact, were Table 3 of Whipps et al. (2006) to be recreated with culture being tested instead of PCR, the results would look very similar. This shows that Whipps et al.'s (2006) 'light infection…' quote should apply not only to PCR but also to culture. This

Table 3. Diagnosis of *Ichthyophonus* infection in Tanana 2004 data (Whipps et al. 2006). C_H: culture test on heart tissue; C_M: culture test on somatic muscle tissue; P_H: PCR test on heart tissue; P_M: PCR test on somatic muscle tissue; H: histology on heart tissue; n: number of samples. Positive (+) and negative (–) diagnosis of *Ichthyophonus* for each test. NA: not assessed; nd: no data

Case	C_H	C_M	P_H	P_M	H	Infection severity	n
A	+	+	+	+	+	Heavy	6
B	+	+	+	+	–	Heavy	1
C	+	+	+	–	+	Heavy	1
D	+	–	+	–	–	Light	2
E	+	–	–	–	–	Light	1
F	–	+	+	–	–	Light	1
G	–	+	–	–	–	Light	2
H	–	–	–	–	–	nd	85
I	–	–	–	–	NA	nd	1
J	+	+	NA	+	+	nd	2
K	+	+	NA	–	+	nd	1
L	+	–	NA	–	+	nd	1
M	+	–	NA	–	–	nd	2
N	–	–	NA	–	+	nd	1
O	–	–	NA	–	–	nd	43
Total							150

is expected, as infection of parasites is not uniformly distributed throughout the host's body (Kocan et al. 2011). Furthermore, *Ichthyophonus* targeting primarily heart muscle does not necessarily mean that the parasite will be found in every heart tissue. When only a small amount of tissue is sampled, some samples from infected fish may not contain *Ichthyophonus* just by chance alone.

In conclusion, we contend that PCR is a viable diagnostic test for *Ichthyophonus*, comparable to culture. Given the advancement of molecular technology, the potential of PCR for advancing epidemiological studies and being used as the main diagnosis tool is high. However, this does not necessarily imply that PCR should replace the culture method. The culture test has been and will remain a standard diagnostic tool. Rather than arguing superiority for one method over the other, we argue that every researcher should acknowledge pros and cons of each diagnostic tool and choose the most appropriate one based on circumstances and study objectives.

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